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Adding hydrogen to the isotopic inventory - Combining $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^2\text{H}$ stable isotope analysis for palaeodietary purposes on archaeological bone

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Abstract

Few papers using hydrogen stable isotope analysis for human palaeodietary reconstruction purposes have been published and the usefulness of this additional dietary indicator is highlighted here. The hydrogen stable isotope results provide evidence for the continued exploitation of aquatic resources throughout the prehistory of the Limfjord area in Denmark, which is supported by the FRUITS estimates using three (CNH) isotopic proxies. While aquatic dietary input has been identified in Mesolithic and Viking Age individuals before, our results show that this in fact continued throughout the periods in between (Neolithic, Bronze and Iron Age), albeit on a small scale.

1. Introduction

The archaeological records in Danish Prehistory suggest a continuation of aquatic exploitation throughout prehistory (Andersen 2008; Jensen 2013; Price 2015). Shell bearing sites have been found from almost all prehistoric periods (Mesolithic, Neolithic, Bronze Age, Iron Age and Viking Age) (Andersen 2008), while fish bones (Enghoff 1999), fishing weirs (Andersen 2007) and hooks are present in varying quantities throughout prehistory (Andersen 2008). Lipid residue analysis from Funnel Beaker pottery revealed that consumption of aquatic resources continued in the Neolithic (Craig et al., 2011), albeit in much smaller quantities compared to the previous period (Schulting 2011). However, stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of human bone collagen from these periods (Tauber 1981; 1983; 1986; Richards *et al.*, 2003; Fischer *et al.*, 2007) does not reveal this continuation of aquatic exploitation and people appear to have lived on a terrestrial-based diet presumably through farming. There are possibly threshold values at work, which need to be exceeded before marine dietary intake becomes visible in the bone collagen stable isotope ratios. For example, marine protein consumption can be masked in terms of $\delta^{13}\text{C}$ values in a low protein diet due to dietary routing (Hedges 2004). In such a diet, dietary carbon can be derived from a combination of carbohydrate, lipid and protein sources rather than protein alone, resulting in more

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isotopically the heaviest plant group (mean $\delta^{13}\text{C}$ values of -12.5‰), while C3 plants produce depleted stable isotopic signals (-27‰) (Smith and Epstein 1971; Price *et al.*, 1985). Although $\delta^{13}\text{C}$ values of marine and C4 plants partially overlap, $\delta^{13}\text{C}$ values can be used here to differentiate between two vegetation types at the base of the food chain, terrestrial C3 (Smith and Epstein 1971; Price *et al.*, 1985) and marine (DeNiro and Epstein 1978), as C4 plants are not expected to have been consumed in prehistoric Denmark. Freshwater fish detection can be successful if there is no overlap between the carbon and nitrogen stable isotope values of consumed foods, e.g. the terrestrial and freshwater sources.

Due to fractionation processes, carbon and nitrogen show an increase in their isotopic ratios of $\sim 1\text{‰}$ (DeNiro and Epstein 1978) and $\sim 3\text{‰}$ (DeNiro and Epstein 1981), respectively, for each trophic level. A nitrogen stable isotope enrichment of 3-4‰ has been accepted from Neolithic to post-Roman times in north-western Europe (Hedges and Reynard 2007), although 5‰ has also been reported (Drucker *et al.*, 2003), and even 6‰ has been suggested (O'Connell *et al.*, 2012). During the turnover of consumed protein in the body, the heavy isotope ^{15}N is preferentially retained in contrast to the preferential excretion of the light isotope ^{14}N (Fry 2006; Hedges and Reynard 2007). As this process intensifies up the food chain, this results in the enrichment of nitrogen stable isotope ratios in human bone collagen.

However, factors other than diet are known to influence stable isotope ratios, such as plant anatomy (Szpak 2014), vegetation cover (Van der Merwe and Medina 1989; 1991), soil composition (Heaton 1987; Britton *et al.*, 2008) and fertilisation practices (Koerner *et al.*, 1999; Bogaard *et al.*, 2007; Szpak 2014). Additionally, a nursing effect can raise $\delta^{15}\text{N}$ values in juvenile animals and subsequently human consumers as well (Fogel *et al.*, 1989; Dürrwächter *et al.*, 2006). And, as mentioned earlier, marine protein consumption can be masked in terms of $\delta^{13}\text{C}$ in a low protein diet (Hedges 2004). In such a diet, the $\delta^{13}\text{C}$ values in bone collagen are less enriched than expected. Additionally, marine fish samples can produce more depleted $\delta^{13}\text{C}$ values depending on origin and feeding habitat (Barrett *et al.*, 2008), which means consumption of such fish would also result in less enriched $\delta^{13}\text{C}$ values than expected.

2.1 Hydrogen

Hydrogen stable isotope analysis is frequently performed on feather and hair samples in forensic investigations (Sharp *et al.*, 2003) and animal migration studies (Nelson *et al.*, 2015), while few publications featuring archaeological human bone material exist (Reynard and Hedges 2008; Arnaud-de-la-Rosa *et al.*, 2010; van der Sluis *et al.*, 2016; Wang *et al.*, 2017). Hydrogen is strongly connected

to precipitation values, which are in turn linked to latitude, altitude and continentality (Meier-Augenstein *et al.*, 2013), although $\delta^2\text{H}$ values also show a good correlation with $\delta^{15}\text{N}$ values and can be considered an additional trophic level indicator (Birchall *et al.*, 2005; Peters *et al.*, 2012; Gröcke *et al.*, 2016). Because $\delta^{15}\text{N}$ values can be influenced by a number of factors other than dietary protein intake, $\delta^2\text{H}$ values can prove to be a useful tool in establishing trophic level. Additionally, considering that marine food chains are generally much longer than terrestrial food chains, $\delta^2\text{H}$ values could potentially also be used to detect marine protein intake. Absolute $\delta^2\text{H}$ values of an individual are linked to geographic location, while the relative difference between consumers increase per trophic level (30-50‰ for herbivores to omnivores and 10-20‰ from omnivores to humans) (Reynard and Hedges 2008). It has been noted that body size can have an effect on $\delta^2\text{H}$ values, resulting in long-term averaged values in large mammals and short-term values in small rodents, potentially reflecting seasonal climate and/or environmental influences (Topalov *et al.*, 2013). $\delta^2\text{H}$ values are controlled by both drinking water and ingested foods, although it is thought that the hydrogen in protein-based tissues is largely derived from the diet rather than from water (Sharp *et al.*, 2003; Bowen *et al.*, 2009). This reduced effect of drinking water on $\delta^2\text{H}$ values might be connected to the slow turnover rate of bone collagen (Topalov *et al.*, 2013).

Hydrogen in bone collagen consists of a non-exchangeable and an exchangeable (~21%) fraction, the latter consisting of labile H atoms that are bound to functional groups, e.g. $-\text{NH}_2$, $-\text{OH}$ and $-\text{COOH}$ (Reynard and Hedges 2008; Meier-Augenstein *et al.*, 2013). This exchangeable fraction equilibrates with hydrogen from the burial and subsequently laboratory environment, resulting in meaningless values. The $\delta^2\text{H}$ values of this fraction need to be determined in order to obtain the $\delta^2\text{H}$ values of the non-exchangeable fraction, which contain the true $\delta^2\text{H}$ values. A comparative equilibration approach can only be applied to material for which a standard exists that is a 100% matrix match. Because such a standard does not exist for bone collagen samples, a 2-stage equilibration method is applied, in which each original sample is divided into two subsamples (A and B), which are equilibrated with two water standards of known isotopic value (Bowen *et al.*, 2005). These two equilibration waters need to differ by at least 100‰. By applying this 2-stage equilibration method, sample specific and process specific factors influencing exchange rates are compensated for and the stable isotope ratio of the exchangeable hydrogen is assumingly fixed (Meier-Augenstein *et al.*, 2011). The equation from Meier-Augenstein and colleagues (2011), which is the rewritten basic equation of $\delta^2\text{H}_{\text{total}} = \delta^2\text{H}_{\text{true}} + \delta^2\text{H}_{\text{exchangeable}}$ with all necessary substitutions (where f_{Hxch} is the molar exchange fraction), can be used to calculate the true $\delta^2\text{H}$ values:

$$\delta^2\text{H}_{\text{true}} = \delta^2\text{H}_{\text{total}} - (f_{\text{Hxch}} \times \delta^2\text{H}_{\text{waterA}}) \quad \text{where} \quad f_{\text{Hxch}} = \frac{\delta^2\text{H}_{\text{sample,waterA}} - \delta^2\text{H}_{\text{sample,waterB}}}{\delta^2\text{H}_{\text{waterA}} - \delta^2\text{H}_{\text{waterB}}}$$

$$(1 - f_{\text{Hxch}})$$

$$\delta^2\text{H}_{\text{waterA}} - \delta^2\text{H}_{\text{waterB}}$$

Standards consist of cold-welded, sealed silver tubes containing water of known isotopic composition (Qi *et al.*, 2010), which are analysed alongside solid samples in the same run. This is crucial in order to prevent scale compression (Meier-Augenstein *et al.*, 2013).

3. Material

In total, 60 (32 human and 28 faunal) bone samples from 45 sites was selected for this study (Appendix 1), (Fig. 1). Most of the human bone samples used in this work are single finds and do not originate from large cemeteries. The majority of the faunal bone material was obtained from the Zoological Museum's collection in Copenhagen, Denmark. Although samples were preferably taken from long bones, samples were occasionally taken from other bones, e.g., cranium, vertebrae and pelvis, when long bones were not available. Adult individuals were sampled when possible to reduce age-related dietary variability.

4. Methods

4.1 Bone collagen extraction

The bone collagen extraction protocol (Brock *et al.*, 2010), which is based on the Longin method (Longin 1971) and revised with the inclusion of an ultrafiltration step (Brown *et al.*, 1988; Bronk Ramsey *et al.*, 2004) was applied. The protocol followed these main steps: bone samples were cleaned with a dremel, demineralised in 10 mL of 2% (0.6M) HCl acid (with acid renewal), rinsed three times with Milli-Q water and gelatinised in weak HCl acid (pH 3) at ~70°C for 24 hours. Samples were Ezee (Elkay®) filtered (60-90µm), Ultrafiltered (30 kDa) for 20 minutes at 3000 RPM, transferred into clean vials, frozen and lyophilised.

4.2 Isotope Ratio Mass Spectrometry (IRMS)

1-1.5 mg of bone collagen was weighed into tin capsules and analysed for their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values using the Thermo Delta V IRMS with Flash 1112 Elemental Analyzer at the $^{14}\text{CHRONO}$ Centre in Queen's University Belfast. Samples were run with standards IA-R041 L-Alanine ($\delta^{15}\text{N}$ -5.56; $\delta^{13}\text{C}$ -23.33), IAEA-N-2 Ammonium Sulphate ($\delta^{15}\text{N}$ = +20.3 ± 0.2) and IAEA-CH-Sucrose ($\delta^{13}\text{C}$ = -10.449 ± 0.033). The standard deviation on the measurements of over 1500 measurements of the IA-R041 L-Alanine ($\delta^{15}\text{N}$ -5.56; $\delta^{13}\text{C}$ -23.33) standard yielded a standard deviation on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of 0.22‰ and 0.15‰, respectively. For $\delta^2\text{H}$ measurements, two subsamples of 0.35-0.40 mg of bone collagen were weighed into silver capsules, crimped into balls and placed within a sealed desiccator

containing 10 mL water of known isotopic composition (Mourne Mountain snow water, Northern Ireland $\delta^2\text{H} = -56.89\text{‰}$ and USGS 49 $\delta^2\text{H} = -394.7\text{‰}$) to equilibrate for 4 days (96 hours) at ambient temperature. After removing the water, samples were dried down for 7 days under a vacuum in sealed desiccators containing silica gel to remove any water vapour. For the first sample batch, 3 samples were taken from the desiccators every 15 minutes and placed in the running Thermo MAS 200R autosampler to reduce exposure to ambient air. The first sample was flushed with helium before being combusted at 1447°C . Following sample batches were analysed using a Costech Zerobank autosampler, enabling the entire carousel to be flushed with 300 mL/min helium for 10 minutes, after which the autosampler was sealed. Both autosampler arrangements were coupled to a Thermo High Temperature Conversion Elemental Analyzer (TC/EA), with a reactor consisting of an outer ceramic mantle tube of aluminium oxide and an inner glassy carbon reactor part-filled with glassy carbon chips. A graphite crucible positioned on these chips received each dropped sample from the autosampler/Zerobank arrangement. For isotopic measurement the TC/EA was coupled with a Thermo Delta V IRMS at the Stable Isotope Facility in the School of Planning, Architecture and Civil Engineering of Queen's University Belfast. To ensure machine integrity, both stability and linearity checks were carried out on the IRMS before each run. Machine precision was 1‰. Samples were calibrated using international standards packed in 0.25 μL silver tubes (Qi *et al.*, 2010), VSMOW ($\delta^2\text{H} = 0\text{‰}$), SLAP-2 ($\delta^2\text{H} = -427.5\text{‰}$) and UC04 ($\delta^2\text{H} = +113\text{‰}$). A run consisted of 12 standards (4 SLAP-2, 4 VSMOW, 4 UC04), 3 Atropina standards (for H% calculation), 2 blanks, 22 samples, followed by 1 blank and 9 standards (3 SLAP-2, 3 VSMOW, 3 UC04). The Atropina standards were used to calculate the H% for quality control purposes, to examine if samples produced similar percentages of hydrogen.

4.3 Accelerator Mass Spectrometry (AMS) and calibration

2.5-3 mg of collagen was loaded with 0.09 g of copper oxide and a silver strip for contaminant removal in a small quartz tube for combustion to CO_2 . Combusted samples were graphitised using a hydrogen reduction method with iron as catalyst. Pressed targets were analysed together with oxalic acid standards and background samples in the NEC compact model 0.5MV AMS at the ^{14}C CHRONO Centre in Belfast. Radiocarbon ages were calculated from $F^{14}\text{C}$ (Reimer *et al.*, 2004), which is corrected for background and isotopic fractionation using $^{13}\text{C}/^{12}\text{C}$ measured by AMS that accounts for both natural and machine isotopic fractionation. An error multiplier of 1.3 was applied to the $F^{14}\text{C}$ measurements to account for variability in sample processing. ^{14}C dates for humans were calibrated using Calib 7.0.2 with the mixed marine (Marine13) and Northern Hemisphere terrestrial (IntCal13) curves (Reimer *et al.*, 2013). Based on 13 known age mollusc measurements (Olsson 1980; Heier-Nielsen *et al.*, 1995) from the Limfjord area from the marine reservoir database

(<http://calib.org/marine/>), the ΔR and uncertainty were calculated ($\Delta R = 239 \pm 164$ yrs). The percentage of marine carbon was calculated using a linear regression between fully marine (-11.8‰) and terrestrial endmembers (-21.5‰) based on $\delta^{13}\text{C}$ values from marine and terrestrial animals. The $\delta^{13}\text{C}$ values of the terrestrial endmembers were calculated using the stable isotope results from 53 herbivore bone samples from various archaeological time periods from the Limfjord area, while the marine endmember values were calculated using the stable isotope results from 27 bone samples from marine animals (van der Sluis 2017).

4.4 Collagen quality criteria

Quality criteria were met to ensure high quality and reliable stable isotope data. The atomic C:N ratio should be between 2.9 and 3.6 (DeNiro 1985), while samples should produce a collagen yield of at least >0.5-1% when ultrafiltered (van Klinken 1999).

4.5 FRUITS Bayesian software

FRUITS is freely available and allows for the inclusion of not only isotopic evidence but also prior information available from other sources such as archaeological records (Fernandes *et al.*, 2016). FRUITS software is described in detail in Fernandes *et al.* (2014). Limitations to the method include possible poorly constrained diet-to-tissue offsets, the lack of isotopic values for all food sources and the difficulty in the establishment of baseline values for edible food as opposed to food remains in archaeological studies (Fernandes *et al.*, 2014).

5. Results

Carbon, nitrogen and hydrogen stable isotope analysis was successfully executed on 60 bone collagen samples (Table 1, Table 2). All samples met the quality criteria. Three collagen yields are unknown, as the vial lids' inside seal was affected by the lyophilisation process. However, these samples visibly produced collagen with acceptable atomic C:N ratios. In all figures the analytical error is shown at the bottom right side. Statistically, there is no significant difference in $\delta^{13}\text{C}$ values between the Neolithic and Bronze Age, or between the Bronze Age and Iron Age, while the $\delta^{13}\text{C}$ values from the Iron Age and Viking Age are significantly different (two-tailed T-test with unequal variances $p = 0.02$, $\alpha = 0.05$). $\delta^{15}\text{N}$ values are statistically the same in the Neolithic and Bronze Age, and the Iron Age and Viking Age, although they differ significantly between the Bronze Age and Iron Age ($p = 0.004$). $\delta^2\text{H}$ values are statistically the same in the Neolithic and Bronze Age ($p = 0.88$), Bronze Age and Iron Age ($p = 0.88$), and the Iron Age and Viking Age ($p = 0.066$).

Table 1. Carbon, nitrogen, hydrogen stable isotope ratios, C:N ratios, collagen yields, conventional and calibrated ¹⁴C ages of the human samples.

Sample number	Sample name	δ ¹³ C (‰) vs PDB	δ ¹⁵ N (‰) vs AIR	Atomic C:N ratio	Collagen yield (%)	δ ² H (‰) vs VSMOW	H (%)	Lab no	¹⁴ C age ±1σ BP	% Marine	¹⁴ C age cal BC/AD (2σ)	Archaeological period
Lim-hb-125	Ertebølle	-14.9	13.3	3.3	4.6	68.3	5.0	UBA-31308	5690 ± 34	49.0	4386-3994 cal BC	Ertebølle
Lim-hb-117	Gudumlund	-10.6	13.1	3.3	8.1	45.8	4.0					Mesolithic
Lim-hb-124	Bjørnsholm Å	-19.8	9.3	3.2	6.7	-10.9	3.3	UBA-31307	4752 ± 37	7.0	3631-3372 cal BC	Early Neolithic
Lim-hb-049	Torsholm	-18.0	8.9	3.3	5	-1.8	5.2	UBA-31955	5038 ± 40	22.4	3799-3535 cal BC	Early Neolithic
Lim-hb-044	Krejbjerg og Ginderup	-18.9	10.1	3.4	2.9	8.5	4.4	UBA-31298	4697 ± 34	14.9	3518-3123 cal BC	Early/Middle Neolithic
Lim-hb-136	Olufshøj	-20.9	8.5	3.3	4.3	-10.4	3.3	UBA-31279	4370 ± 50	-2.3	3323-2897 cal BC	Middle Neolithic
Lim-hb-004	Bjørnsholm Vitskøl	-20.2	8.5	3.3	-	-12.2		UBA-31296	4303 ± 43	3.1	3022-2760 cal BC	Middle Neolithic/SGK
Lim-hb-095	Øslev	-20.4	8.6	3.2	6.4	0.7	4.2					Single Grave Culture
Lim-hb-131	Øster Harup	-20.1	8.6	3.2	12.1	3.4	4.6					Single Grave Culture
Lim-hb-052	Ørnshøj	-19.1	8.0	3.2	-	9.3	4.7	UBA-31306	3640 ± 54	13.1	2120-1744 cal BC	Late Neolithic
Lim-hb-132	Sejerslev (Brunhøj)	-20.1	10.8	3.2	5.5	-5.0	3.5					Late Neolithic/EBA
Lim-hb-002	Bersted	-19.5	9.5	3.2	20.9	6.9	4.6					Early Bronze Age
Lim-hb-042	Følhøj	-20.2	8.9	3.3	13.4	4.8	4.5					Early Bronze Age
Lim-hb-143	Jestrup	-19.9	10.4	3.2	11	-4.5	4.3	UBA-31283	3114 ± 40	5.7	1431-1231 cal BC	Early Bronze Age
Lim-hb-050	Nors	-20.2	9.6	3.3	5.7	-16.8						Early Bronze Age
Lim-hb-027	Nytorp	-20.1	9.4	3.3	5.6	-10.3						Early Bronze Age
Lim-hb-028	Nytorp	-19.6	8.8	3.2	9.3	6.7	5.2					Early Bronze Age
Lim-hb-128	Sebber Skole #3	-19.5	8.7	3.3	7.9	1.3	4.2					Early Bronze Age
Lim-hb-140	Sennels	-18.9	12.4	3.2	5.4	-9.3	5.3	UBA-31281	2562 ± 59	14.5	775-415 cal BC	Late Bronze/Pre-Roman IA
Lim-hb-177	Stubberup	-21.1	10.9	3.2	12.7	7.2	5.3	UBA-31276	2159 ± 50	-3.9	381-102 cal BC	Pre-Roman Iron Age
Lim-hb-007	Hamahus	-19.4	11.1	3.2	12.3	14.3	5.2					Early Roman Iron Age
Lim-hb-110	Nørre Tranders grusgrav	-20.3	11.7	3.4	7.2	-3.1	5.1					Early Roman Iron Age
Lim-hb-105	Rygegård	-20.3	10.9	3.3	3.6	3.5	5.1	UBA-31303	1869 ± 28	2.8	85-232 cal BC	Early Roman Iron Age

Sample number	Sample name	$\delta^{13}\text{C}$ (‰) vs PDB	$\delta^{15}\text{N}$ (‰) vs AIR	Atomic C:N ratio	Collagen yield (%)	$\delta^2\text{H}$ (‰) vs VSMOW	H (%)	Lab no	^{14}C age $\pm 1\sigma$ BP	% Marine	^{14}C age cal BC/AD (2 σ)	Archaeological period
Lim-hb-147	Dragsbæk	-20.4	12.2	3.4	1.4	-19.2	3.7					Early Roman Iron Age
Lim-hb-159	Beersted bæk	-20.4	12.4	3.2	13.1	-13.3	5.3					Early Iron Age
Lim-hb-103	Sejlfjord	-19.8	12.7	3.3	2.5	-7.1	5.1					Germanic Iron Age
Lim-hb-173	Grundvad Bæk	-20.2	11.9	3.2	6.5	10.0	5.3	UBA-31273	1252 \pm 52	3.7	cal AD 668-941	Germanic/Viking Age
Lim-hb-031	Romb	-20.5	11.7	3.6	-	10.0	5.3	UBA-31300	1259 \pm 29	1.1	cal AD 675-867	Germanic/Viking Age
Lim-hb-001	Aggersborg Kirke	-18.8	12.4	3.2	11.4	34.4	5.4					Viking Age
Lim-hb-022	Brårup	-20.3	10.7	3.3	-	-13.6						Viking Age
Lim-hb-085	Sebbersund	-18.1	12.9	3.3	12.1	33.3	5.1					Viking Age
Lim-hb-086	Sebbersund	-17.9	12.9	3.2	12.5	30.2	4.4					Viking Age
Lim-hb-089	Sebbersund	-17.5	13.9	3.2	10.5	48.8						Viking Age

Table 2. Carbon, nitrogen, hydrogen stable isotope ratios, atomic C:N ratios and collagen yields of the faunal samples.

Sample number	Species name	$\delta^{13}\text{C}$ (‰) vs PDB	$\delta^{15}\text{N}$ (‰) vs AIR	Atomic C:N ratio	Collagen yield (%)	$\delta^2\text{H}$ (‰) vs VSMOW	H (%)	Archaeological period
Lim-ab-241	<i>Bos primigenius</i>	-23.2	4.3	3.2	15	-48.5	5.3	Ertebølle
Lim-ab-244	<i>Canis familiaris</i>	-10.9	11.5	3.2	11.3	84.7	4.9	Ertebølle
Lim-ab-250	<i>Canis familiaris</i>	-12.7	13.1	3.2	8.1	68.9	5.0	early Neolithic
Lim-ab-257	<i>Bos taurus</i>	-21.7	4.4	3.2	8.3	-46.1	5.0	early Neolithic
Lim-ab-248	<i>Bos taurus</i>	-21.2	5.7	3.2	12.5	-45.8	5.1	Neolithic
Lim-ab-323	<i>Sus scrofa</i>	-21.6	4.0	3.3	8.8	-29.4	5.0	Neolithic
Lim-ab-325	<i>Halicoerus gryphus</i>	-11.7	17.5	3.2	4.3	118.9	4.9	Neolithic
Lim-ab-326	<i>Halicoerus gryphus</i>	-8.8	14.2	3.2	5.4	144.1	4.8	Neolithic
Lim-ab-207	<i>Ovis/Capra</i>	-22.1	7.7	3.4	5.3	-39.1	5.3	Early Bronze Age?
Lim-ab-222	<i>Bos taurus</i>	-20.9	4.2	3.4	2.7	-39.5	4.9	Bronze Age
Lim-ab-223	<i>Ovis aries</i>	-21.3	5.5	3.2	12.1	-44.7	5.3	Bronze Age

Lim-ab-227	fish	-11.8	14.6	3.1	5	15.8	4.5	Bronze Age
Lim-ab-320	<i>Orcinus orca</i>	-11.7	18.7	3.2	13.2	216.5	4.7	Bronze Age
Lim-ab-200	herbivore	-21.1	4.4	3.2	7.2	-53.2	4.3	Bronze Age/Iron Age
Lim-ab-270	fish	-7.3	7.3	3.3	9.8	-14.5	5.0	Pre-Roman Iron Age
Lim-ab-315	<i>Ovis/Capra</i>	-21.5	9.3	3.3	12.9	-43.3	4.9	Early Iron Age
Lim-ab-316	<i>Bos taurus</i>	-21.3	5.1	3.3	3.6	-61.7	3.2	Early Iron Age
Lim-ab-199	<i>Ovis/Capra</i>	-22.0	6.9	3.2	8.2	-47.9	5.0	Iron Age
Lim-ab-290	<i>Belone belone</i>	-13.0	13.4	3.1	12.1	129.1	4.2	Iron Age
Lim-ab-294	<i>Ovis/Capra</i>	-21.4	5.9	3.2	6.9	-43.4	4.8	Iron Age
Lim-ab-329	<i>Belone belone</i>	-12.8	12.4	3.2	7.9	101.7	5.0	Iron Age
Lim-ab-212	<i>Belone aces</i>	-13.0	12.8	3.1	10.9	122.8	5.1	Viking Age
Lim-ab-275	fish	-9.3	7.5	3.1	9.3	1.7	5.0	Viking Age
Lim-ab-234	<i>Bos taurus</i>	-21.6	5.4	3.2	10.1	-51.9	5.3	Viking Age/early medieval
Lim-ab-235	<i>Sus domesticus</i>	-21.5	9.4	3.2	7.3	-33.2	5.2	Viking Age/early medieval
Lim-ab-236	<i>Gadid sp.</i>	-13.9	15.1	3.2	5.3	34.1	3.6	Viking Age/early medieval
Lim-ab-280	<i>Bos taurus</i>	-21.8	7.4	3.2	14.2	-46.1	5.3	Viking Age/Early medieval
Lim-ab-286	<i>Sus domesticus</i>	-21.6	10.7	3.3	12.8	-12.3	5.4	Viking Age/Early medieval

samples.

5.1 Carbon and nitrogen stable isotope results

Although a few animal samples were recorded as juvenile or subadult, none of them showed a trophic level enrichment, except for one Viking Age pig ($\delta^{15}\text{N} = 9.4\text{‰}$) (Fig. 2B). However, there is another pig with an even higher $\delta^{15}\text{N}$ value (10.7‰) that has not been recorded as juvenile. This pig sample has not been absolutely dated but originates from a city excavation in Skive and is probably Viking Age/Early Medieval. Since both these pig samples are from relatively young archaeological periods, their high $\delta^{15}\text{N}$ values could be temporally related. While Mesolithic and Neolithic faunal samples generally seem to be characterised by low isotopic ratios, there does not appear to be a chronological trend in the elevation of the stable isotope ratios throughout the millennia, i.e. not all Iron Age cattle have higher $\delta^{15}\text{N}$ values than Neolithic cattle. One human individual was recorded as juvenile and produced elevated stable isotope ratios in comparison to the other Bronze Age individuals, suggesting a nursing effect (Lim-hb-140 with $\delta^{13}\text{C} = -18.9\text{‰}$, $\delta^{15}\text{N} = 12.4\text{‰}$, $\delta^2\text{H} = -9.3\text{‰}$) (Fig. 2D). The calibrated radiocarbon age (766-401 cal BC) of this sample obtained from the radiocarbon age from museum documents covers the Late Bronze Age and the first 100 years of the following period. As such, this sample could also stem from the Pre-Roman Iron Age.

5.2 Hydrogen stable isotope results

The analysed subsamples, calculated non-exchangeable $\delta^2\text{H}$ values and the fHxch value are presented (Appendix 2). The $\delta^2\text{H}$ values in human samples are poorly correlated with the $\delta^{15}\text{N}$ values ($R^2 = 0.29$) (Fig. 2B), yet moderately correlated with the $\delta^{13}\text{C}$ values ($R^2 = 0.55$) (Fig. 2C). When human and animal samples are combined, the $\delta^2\text{H}$ values still show a moderate correlation with the $\delta^{13}\text{C}$ values ($R^2 = 0.55$), yet a higher correlation is visible with the $\delta^{15}\text{N}$ values ($R^2 = 0.63$). The Pearson coefficient is used here since it holds for non-normal distributions. Considerable variation in the $\delta^2\text{H}$ values of duplicate measurements (0.1-11.5‰) was observed in the first batch of 10 samples, after which it was decided to homogenise the samples using mortar and pestle. Samples were run in duplicates, which generally gave very little variation (<2‰), although some samples still had more variable hydrogen isotope ratios (<5 ‰). Six samples had variation of just over 5‰ (5.2‰, 5.2‰, 5.1‰, 5.1‰ and 5.3‰), while 5 samples gave variation between 6-8‰ and one sample gave 10.3‰. This larger variation was usually encountered in samples with very fluffy white collagen, which was difficult to homogenise. In order to investigate whether any underlying trends were present in the stable isotope data, a Principal Component Analysis (PCA) was performed, which revealed that 80.4% of the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^2\text{H}$ data can be explained with the first component (F1), while F2 explains another 14.3%,

meaning that F1 and F2 together explain 94.7% of the data. This would suggest that all three stable isotope values correlate to some extent due to a single underlying factor they all have in common, such as trophic level enrichment. Any deviations from F1 could be explained by factors that may affect one ratio but not the other two, such as the use of fertilisers, which will affect $\delta^{15}\text{N}$ values, and latitudinal differences/precipitation, which would affect $\delta^2\text{H}$ values but not $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. As 80% of the data can be explained by one factor, there does not appear to be a hidden underlying trend.

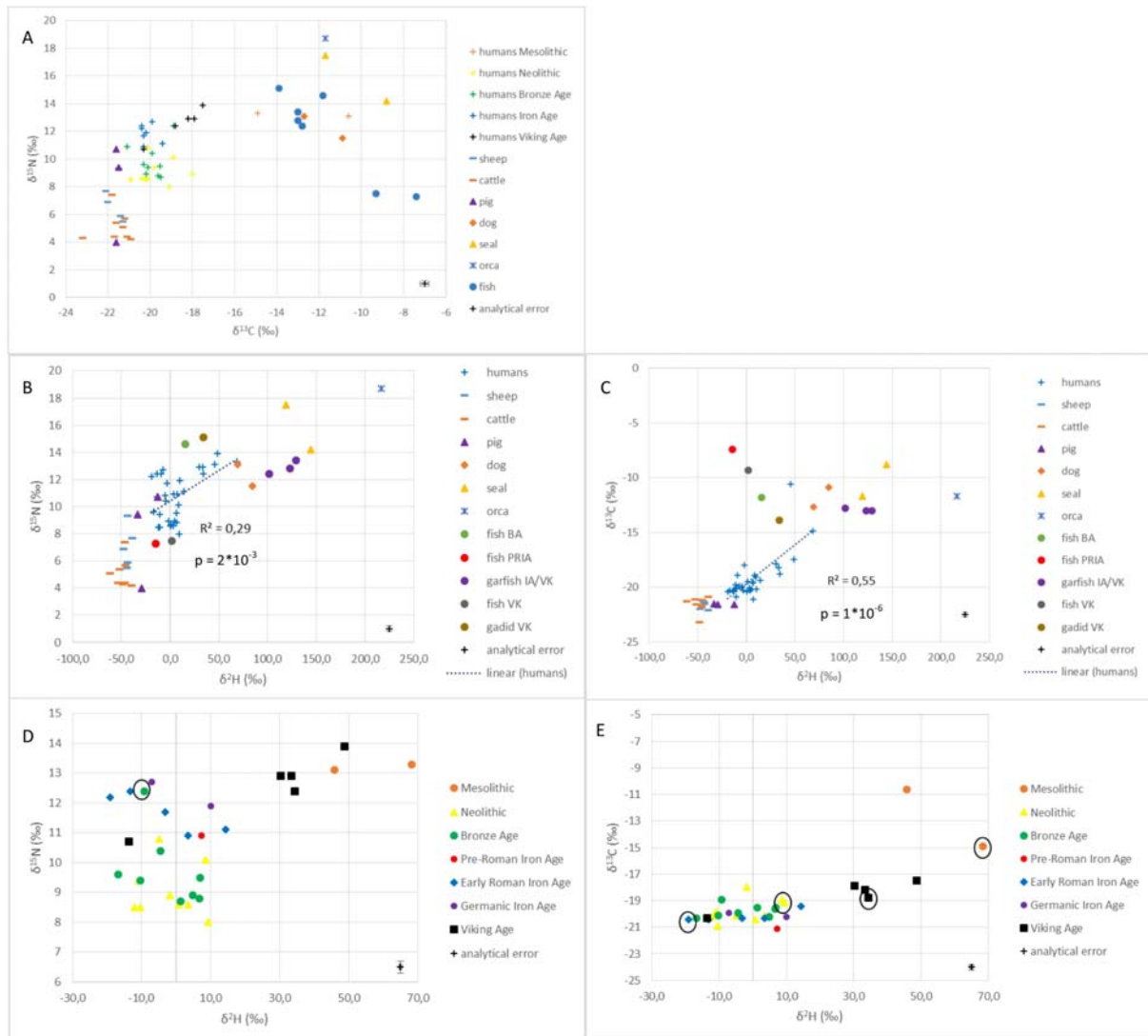


Figure 2. Overview of the stable isotope results. A: $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of human and animal samples. B: $\delta^2\text{H}$ and $\delta^{15}\text{N}$ values of human and animal samples with the linear regression line for the human samples. C: $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of human and animal samples with the linear regression line for the human samples. D: $\delta^2\text{H}$ and $\delta^{15}\text{N}$ values of human samples per archaeological period. The circled Bronze Age individual shows a potential nursing effect (the sample could also be Pre-Roman Iron Age). E: $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values of human samples per archaeological period. The circled individuals were selected for modelling with FRUITS.

5.3 Radiocarbon results

A total of 12 human bone samples were AMS ^{14}C dated. Both conventional radiocarbon ages (^{14}C age BP) and calibrated ages (cal BC at 2σ) are presented (Table 1). The percentage of marine protein in the diet is calculated using a linear regression between a terrestrial (-21.5‰) and 100% marine (-11.8‰) endmember for $\delta^{13}\text{C}$ value, to which a 1‰ diet to consumer offset is applied.

6. Discussion

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the human individuals from the Limfjord are discussed, after which the use of hydrogen stable isotope ratios is discussed, followed by a section in which dietary components are quantified for several individuals using the software FRUITS.

6.1 Assessing the Limfjord's human diets

While Mesolithic and Viking Age individuals display elevated $\delta^{13}\text{C}$ values, indicating various quantities of marine protein consumption, $\delta^{13}\text{C}$ values from Neolithic, Bronze Age and Iron Age individuals suggest people were mainly living on a diet based on terrestrial C3 plants and/or animals consuming C3 plants. The dichotomy in $\delta^{13}\text{C}$ values between the two Mesolithic human samples (Fig. 2E) is similar to Craig et al.'s (2006) observation on dog and seal samples from southern Scandinavia. Individuals with lower $\delta^{13}\text{C}$ values, around -16‰, possibly consumed aquatic food sources that were influenced by freshwater from the Baltic. The radical difference in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between Mesolithic and Neolithic samples corresponds to the transition from a hunting-gathering-fishing lifestyle to a farming lifestyle. Neolithic individuals reveal stable isotope ratios suggesting a diet based on terrestrial sources with potentially small additions of freshwater fish. While the Bronze Age diet was isotopically similar to the Neolithic diet, an elevation in $\delta^{15}\text{N}$ values is discernible, combined with terrestrial $\delta^{13}\text{C}$ values (between -20‰ and -20.5‰), in the following periods of the Roman Iron Age. It is not until the Viking Age that an increase in $\delta^{13}\text{C}$ values becomes apparent again. Possible explanations for elevated $\delta^{15}\text{N}$ values in the Iron Age are consumption of a diet based on plants grown on soils high in ^{15}N as a result from fertilising (Kanstrup et al., 2014), a diet based on livestock feeding on soils with plants high in ^{15}N , such as salt marshes (Britton et al., 2008; Müldner et al., 2014), and/or freshwater fish consumption (Schoeninger and DeNiro 1984; Schoeller et al., 1986; Dürrewächter et al., 2006). Kanstrup et al. (2014) found that $\delta^{15}\text{N}$ values in archaeological cereal grains ranged between 2‰ and 8‰, and averaged at 6‰. A diet based on cereal grains with $\delta^{15}\text{N} = 6‰$ would result in $\delta^{15}\text{N} = 9\text{-}10‰$ in humans using a 3-4‰ diet to consumer offset. A cereal-based diet could have been the case for Neolithic and Bronze Age individuals. In order to explain the elevated $\delta^{15}\text{N}$ values of 10-12‰ in Iron Age individuals from the Limfjord by consumption of manured cereal grains, a diet based on cereal grains with $\delta^{15}\text{N} = 8‰$ would need to have been consumed consistently.

Of the 48 analysed naked barley samples, eight samples produced $\delta^{15}\text{N} > 6\text{‰}$, and Kanstrup et al., also pointed out that large variation in $\delta^{15}\text{N}$ values (1.5-8‰) in naked barley is possible. It seems unlikely that the elevated $\delta^{15}\text{N}$ signal in the Iron Age humans can be explained entirely by consuming cereal grains enriched in ^{15}N due to manuring and it is likely that other factors influencing $\delta^{15}\text{N}$ values are at play. Hydrogen can aid in finding the answer to the elevated $\delta^{15}\text{N}$ values in the Iron Age individuals. If Iron Age humans were consuming mainly plants or livestock characterised with elevated $\delta^{15}\text{N}$ values due to manuring or salt marsh grazing, their trophic level and $\delta^2\text{H}$ values would be similar to Neolithic and Bronze Age individuals, while freshwater and marine fish consumption would result in a trophic level enrichment and thus also higher $\delta^2\text{H}$ values.

6.2 Hydrogen

Although a number of cows and sheep have varying $\delta^{15}\text{N}$ values, they all show similarly low $\delta^2\text{H}$ values (Fig. 2B). This suggests there is a factor other than diet influencing the $\delta^{15}\text{N}$ values, possibly manuring. The same is visible in two of the three pig samples. However, the pig with the highest $\delta^{15}\text{N}$ value (10.7‰), also has a higher $\delta^2\text{H}$ value than the other two pig samples. This sample is Viking Age/Early medieval and it is likely that this particular pig did have some marine protein in its diet, since its $\delta^2\text{H}$ value is circa 20‰ higher than the two herbivorous pigs. It is unlikely that the age of the sample plays a role, since the other two samples that produced similar $\delta^2\text{H}$ values are Neolithic and Viking Age. The fish samples reveal large variation in their $\delta^2\text{H}$ values, although the garfish from the Viking Age cluster together quite tightly. They also produced high $\delta^2\text{H}$ values, very similar to one of the seals. The orca produced the very high $\delta^2\text{H}$ value of 216‰, underlining the orca's position as top predator in the marine food chain. The $\delta^2\text{H}$ values of human samples are spread out between the terrestrial and marine animals. Two Mesolithic human samples show elevated $\delta^2\text{H}$ values, 45‰ and 68‰, although the sample with the highest $\delta^{13}\text{C}$ value (-10.6‰) did not produce the highest $\delta^2\text{H}$ value (Fig. 2E). This suggests the $\delta^{13}\text{C}$ value can be influenced by other factors, most likely through the consumption of marine animals with varying $\delta^{13}\text{C}$ value, which are in turn related to freshwater input from the Baltic. Human samples do not show any overlap with the herbivorous pigs in terms of their $\delta^2\text{H}$ values, but do show overlap with the pig that likely has some marine protein in its diet. In addition to this, there are fish samples that also show overlap with humans in terms of their $\delta^2\text{H}$ values. This suggests that humans, even with the lowest $\delta^2\text{H}$ values, could have consumed some fish protein, albeit likely in small quantities.

The spread in $\delta^2\text{H}$ values in human samples cannot simply be explained as being related to archaeological time periods, as Neolithic, Bronze Age and Iron Age samples largely overlap (Fig. 2D, Fig. 2E). This suggests that the $\delta^2\text{H}$ values in this region are not governed by precipitation or climate

regimes in the different prehistoric times. Instead, they do seem to be connected to dietary changes. This also means that most humans in these three periods are of similar trophic level, although there is individual variation in people's diet in each archaeological period. While the $\delta^{15}\text{N}$ values show a rise in human samples from the Bronze Age to the Iron Age in the Limfjord, suggesting a dietary shift, the $\delta^2\text{H}$ values reveal a different story. Individuals from various archaeological periods overlap in their $\delta^2\text{H}$ values, which suggests that there is not a trophic level shift associated with the rise in $\delta^{15}\text{N}$ values in Iron Age people. Additionally, the absence of a similar rise in $\delta^{15}\text{N}$ values in Iron Age livestock (van der Sluis 2017) would suggest that this trend is only related to the human diet, most likely caused, in part, by manuring of the agricultural fields that were in use for the production of crops for human consumption. As such, there were two main results obtained from the $\delta^2\text{H}$ values: the $\delta^2\text{H}$ values revealed that the $\delta^{15}\text{N}$ values in Iron Age humans are influenced, probably due to manuring, but they also show that fish was consumed throughout prehistoric times in varying quantities.

6.3 Quantifying food sources using Bayesian software FRUITS

The wide range in the $\delta^2\text{H}$ values of the humans from various archaeological periods suggests varying quantities of higher trophic level food (fish) were added to the diet (Fig. 2D, Fig. 2E). In an attempt to quantify particular food sources in the palaeodiet, the stable isotope ratios were entered in a mixing model called FRUITS (Fernandes *et al.*, 2014). FRUITS software runs on Bayesian statistics and is able to calculate probability distributions relating to consumed diets. Three food sources were used (Table 3). The plant data is obtained from Kanstrup *et al.* (2014), who showed that $\delta^{15}\text{N}$ values in cereal grains ranged between 2-8‰ and averaged at 6‰ in prehistoric Denmark. However, the analysed Neolithic plant material produced lower $\delta^{15}\text{N}$ values (4‰) (Kanstrup *et al.*, 2014), which will be tested here as well. The average $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^2\text{H}$ value of cattle, sheep and pigs was used for the terrestrial meat endpoint, while the averages of all marine fish and mammals were combined for the marine endpoint. Because no measured $\delta^2\text{H}$ values of Danish plant material are available, the $\delta^2\text{H}$ values for plant material are estimated at a trophic level below the herbivores. As Reynard and Hedges (2008) observed a shift of 30-50‰ from herbivores to omnivores, the shift here is set to 40‰ with a large uncertainty ($\pm 20\%$) (Table 3). Uncertainties for consumer data were set to 1‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, and 5‰ for $\delta^2\text{H}$ values. Diet to collagen offsets were set to $5 \pm 2.2\%$, $5.5 \pm 0.5\%$ (similar to Fernandes 2016) and $40 \pm 10\%$ for CNH, respectively. Food concentrations were set to 100% because we are using a single fraction (protein). No prior information was included. The estimates are presented as probability distributions (Fig. 3). It is important to underline the absence of freshwater and shell fish samples in this model as these sources were likely to have been consumed as well.

Table 3. Stable isotope ratios used as food sources in FRUITS.

Food source	$\delta^{13}\text{C}$ ratio	$\delta^{15}\text{N}$ ratio	$\delta^2\text{H}$ ratio
Plant	-27‰	4/6/8‰	-82.9‰
Terrestrial meat	-21.6‰	6.3‰	-42.9‰
Marine fish	-11.3‰	13.4‰	87.0‰

Using the stable isotope values from the food sources (Table 3) and assuming that few, if any, of the humans were recent migrants at time of death then we estimate the average protein component of the diet for the region for each time period. The average Neolithic protein dietary intake ($\delta^{13}\text{C} = -19.7\text{‰}$, $\delta^{15}\text{N} = 9.0\text{‰}$, $\delta^2\text{H} = -2.0\text{‰}$) consisted of 57% plants, 34% meat and 9% fish (Fig. 3A) using plant $\delta^{15}\text{N} = 4\text{‰}$. Using a higher $\delta^{15}\text{N}$ value for the plant material (6‰), the estimates change to 50% plant, 44% meat and 6% fish (Fig. 3B). The almost equal amounts of plant and meat protein in the diet are most likely due to similarities in the $\delta^{15}\text{N}$ value. Important to note is that the small scale aquatic dietary input remains present, regardless of the fertilisation effect on the $\delta^{15}\text{N}$ value. With the same food source isotope values, the average Iron Age protein dietary intake ($\delta^{13}\text{C} = -20.2\text{‰}$, $\delta^{15}\text{N} = 11.7\text{‰}$, $\delta^2\text{H} = -3.6\text{‰}$) consisted of 51% of plants, 39% meat and 10% fish (Fig. 3C). Estimates for the Iron Age change to 37% plant, 54% meat and 8% fish, when the $\delta^{15}\text{N}$ value of plants is set to 8‰. Interestingly, the modelled amount of protein from fish in the diet does not change when the $\delta^{15}\text{N}$ value for plants is set to 8‰ (Fig. 3D), only the quantity of protein consumed meat and plant changes.

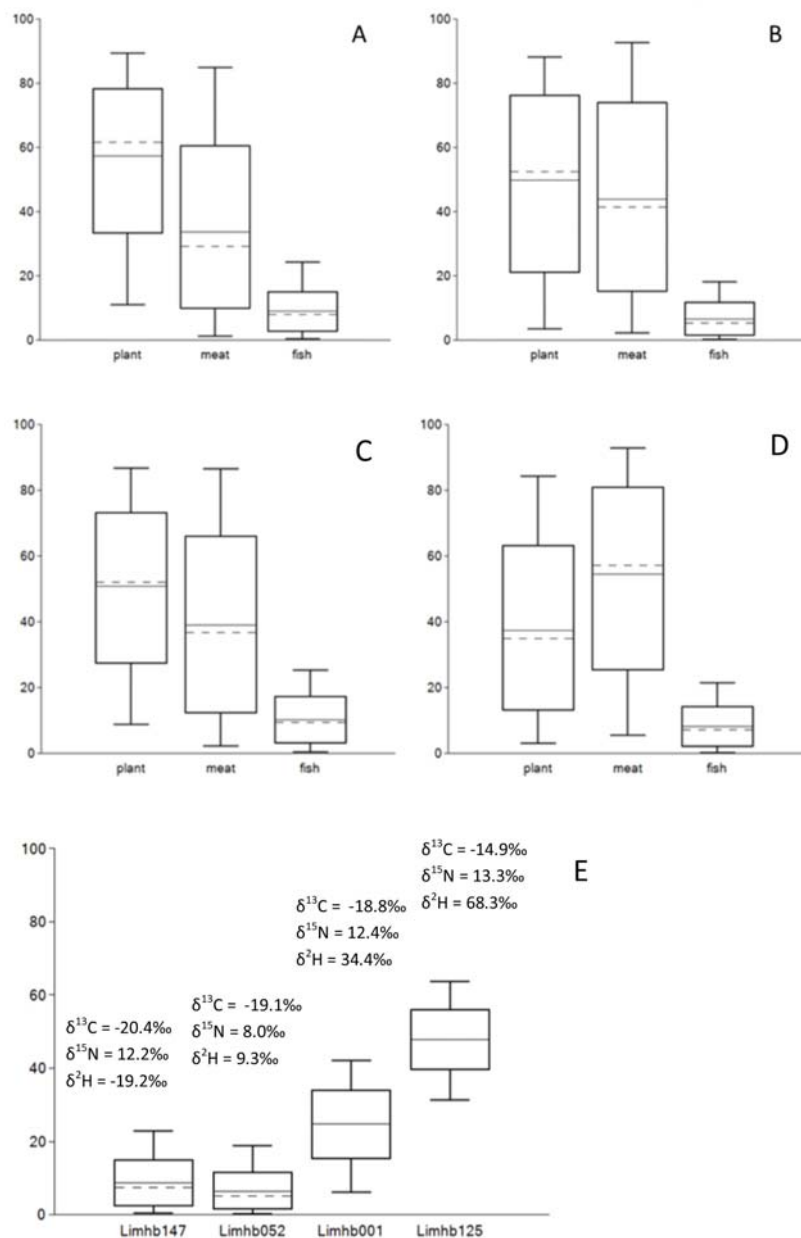


Figure 3. The estimated amounts of food source in model outputs. The x-axis displays the sources, while the y-axis indicates source quantity. Boxes represent 68% credibility intervals, the solid line in the boxes represents the average, the dashed line represents the median value, while the whiskers represent 95% credible intervals. A+B: The average Neolithic diet using three sources and three proxies (CNH). A: plant $\delta^{15}\text{N} = 4\text{‰}$, B: $\delta^{15}\text{N} = 6\text{‰}$. C+D: The average Iron Age diet using three sources and three proxies (CNH) C: plant $\delta^{15}\text{N} = 6\text{‰}$, D: $\delta^{15}\text{N} = 8\text{‰}$. E: Estimated quantity of fish consumption for four individuals (Iron Age (147), Neolithic (052), Viking Age (001) and Mesolithic (125)) using three sources and three proxies (CNH). Plant $\delta^{15}\text{N} = 6\text{‰}$.

Quantification of plant, meat and fish protein in the diet was estimated for 4 individuals: (Lim-hb-147 from the Iron Age, Lim-hb-052 from the Neolithic, Lim-hb-001 from the Viking Age and Lim-hb-125 from the Mesolithic). These samples were chosen based on the distribution of their hydrogen isotope

ratios (Fig. 2E). The outcomes were as follows: the Neolithic (Lim-hb-147) individual had a dietary protein intake consisting of 58% plants, 33% meat and 9% fish, which was 37% plants, 57% meat and 6% fish in the Iron Age (Lim-hb-052) individual, 34% plants, 41% meat and 25% fish in the Viking Age (Lim-hb-001) individual, and 22% plants, 30% meat and 48% fish in the Mesolithic (Lim-hb-125) individual. The reason that Lim-hb-147 (Iron Age) and Lim-hb-052 (Neolithic) have similar estimates of fish protein in their diets is most likely related to the high $\delta^{15}\text{N}$ value in the Iron Age sample (Fig. 3E). Both individuals' diets consisted of circa 9% and 6% of fish protein, respectively. An increase in the amount of consumed fish protein between the Neolithic (Lim-hb-052) and Viking Age (Lim-hb-001) individuals is probably related to the difference in hydrogen and nitrogen isotope values, since their carbon isotope ratios are very similar (Fig. 2E). The Viking Age individual had an average of 25% of their dietary protein intake consisting of fish, while this was 48% for the Mesolithic individual.

The hydrogen stable isotope measurements support the existing archaeological evidence, namely that people were known to be farmers (based on houses, settlement patterns, ploughed fields) and also used animal husbandry (based on finds of faunal bone remains) alongside their agricultural practices (ploughing marks, fields systems). However, it has been difficult to prove that people were also exploiting aquatic resources, not only in the Iron Age but other archaeological periods as well. While fish bones and shell middens were present in varying sizes and amounts throughout all prehistoric periods, it has been difficult to quantify these dietary resources. One reason is that shell middens disproportionally reflect aquatic consumption, i.e. for a relatively small caloric intake a lot of shells are left behind. The hydrogen stable isotope measurement in this study prove that fish were present in people's diet throughout prehistoric times, which is supported by the FRUITS model outcomes. The varying quantities of aquatic protein shown to be supplementing the diet could be related to different types of fish, e.g. low trophic level fish and shellfish. Varying quantities of fish consumption were not related to specific time periods but more to individual variation.

7. Conclusion

The $\delta^2\text{H}$ measurements were instrumental in this work to find isotopic evidence for the continued exploitation of aquatic resources throughout the prehistory of the Limfjord. Additionally, the hydrogen stable isotope results were key to identify the nature of the elevated $\delta^{15}\text{N}$ values in Iron Age individuals. This study exemplified the value of hydrogen stable isotope analysis and the new information that can be obtained by adding it to the isotopic inventory. Using our average stable isotope values from terrestrial and marine animals and a literature value for terrestrial plants as input in the FRUITS model we were able to estimate the diets of humans for different periods (Mesolithic 22% plants, 30% meat and 48% marine fish; Neolithic 58% plants, 33% meat and 9% marine fish; Iron

Age 37% plants, 57% meat and 6% marine fish; Viking Age 34% plants, 41% meat and 25% marine fish).

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